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Docket No. 0174-4002US2 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Jutila, Mark A.	Group Unit: 1806
Serial No.	:	08/463,707	Examiner: Phillip Gambel
File	:	June 5, 1995	
For	:	ANTIBODIES WITH SPECIFICITY FOR A COMMON EPITOPE ON SELECTIN MOLECULES	

Assistant Commissioner of Patents
Washington, D.C. 20231

Declaration Under 35 U.S.C. §1.132
By Takashi Kei Kishimoto

Sir:

I, Takashi Kei Kishimoto declare that:

1. I am a principal scientist at Boehringer Ingelheim Pharmaceuticals, Inc. in Ridgefield, Connecticut.

2. I received a B.A. degree in Biology from New College in 1983. I received a Ph.D. degree in Immunology from Harvard University in 1988.

3. My position, society memberships and bibliography are detailed in the enclosed curriculum vitae (Exhibit A).

4. I have authored over 40 scientific publications related to the study of adhesion molecules and to monoclonal antibodies to selectins and other adhesion molecules.

5. I have read the disclosure of the invention and the pending claims in the patent application Serial No. 08/463,707 ('707).

6. I am making this declaration in support of the allowance of the '707 patent application.

7. I am a coauthor, along with E.C. Butcher and Mark A. Jutila, of the article entitled, Identification of a Human Peripheral Lymph Node Homing Receptor: A Rapidly Down-regulated Adhesion Molecule, Proc. Natl. Acad. Sci USA Vol. 87, pp. 2224-2248, 1990 (referred to hereafter a Kishimoto et al PNAS).

8. The Kishimoto et al PNAS publication discloses the production and characterization of five monoclonal antibodies referred to therein as down-regulated antigens (DREG) monoclonal antibodies, i.e. DREG -55, -56, -110, -152 and -200.

9. I was directly involved in the production of the DREG antibodies and the characterization of the DREG antibodies.

10. The immunogen disclosed in Kishimoto et al PNAS to elicit the DREG antibodies was a shed human leukocyte surface antigen contained in culture supernatants of human peripheral blood leukocytes activated with phorbol myristate acetate (PMA).

11. All of the DREG antibodies elicited using this immunogen specifically recognize the shed or soluble form of L-selectin.

12. The DREG antibodies do not react with human E-selectin.

13. In contrast to the DREG monoclonal antibodies, the monoclonal antibodies of the present invention do not react with the soluble, shed form of L-selectin.

14. The following is a summary of the factual evidence that an antibody exemplified in the present invention as EL-246 does not recognize the soluble or shed form of L-selectin. The EL-246 antibody referred to in the following is identical to the EL-246 antibody disclosed and claimed in the patent application. A sample of the EL-246 antibody was given to me by Mark A. Jutila. The data presented herein was generated by my technicians, Julius Kahn and Grace Migaki, under my direct supervision in my laboratory at Boehringer Ingelheim.

15. Previous results from Dr. Jutila indicated the DREG-200 and EL-246 recognize distinct epitopes on L-selectin, and that the two MAbs do not cross-block each other. A trapping ELISA was set up using biotinylated DREG-200 for the detection of soluble L-selectin, and EL-246 versus DREG-55 was compared as a trapping MAb for soluble L-selectin. A recombinant soluble form of human L-selectin was used in this assay. This recombinant soluble form of L-selectin is structurally equivalent to the shed form of L-selectin released from PMA activated human peripheral leukocytes. The shed form of human L-selectin has been shown to be approximately 6kD smaller than the cell surface form and lacks the cytoplasmic tail and transmembrane domain of the molecule (Kahn, J., R.H. Ingraham, F. Shirley, G. Migaki and T.K. Kishimoto 1994. Membrane Proximal Cleavage of L-selectin: Identification of the Cleavage Site and a 6KD Transmembrane Peptide Fragment of L-selectin. J. Cell Biol. 125: 461-470 - Exhibit B). The recombinant soluble form of L-selectin, like the shed form, lacks the cytoplasmic tail and transmembrane domain of the molecule. All of the DREG antibodies disclosed in Kishimoto et al PNAS bind to the recombinant soluble form of human L-selectin.

COS cells were mock transfected or transiently transfected with a cDNA clone encoding a recombinant soluble form of L-selectin, as indicated, and cell-free supernatants were harvested. 96-well microtiter plates were coated with 1 μg of DREG-55 anti-L-selectin antibody, with 1 μg of EL-246, or with 1 μg of negative control CL37 (anti-E-selectin) MAb, as indicated. Recombinant soluble L-selectin was incubated with the various trapping MAbs. The plates were washed to remove unbound L-selectin, and a control biotinylated MAb, CL2 (anti-E-selectin), or biotinylated-DREG-200 (anti-L-selectin) was added, as indicated (Exhibit C - Figure 1 - EL-246 vs. DREG-55 as a trapping MAb). Biotinylated antibodies bound to the trapped L-selectin was detected by an streptavidin-peroxidase conjugate and an ABTS substrate. Plates were read on a spectrophotometer at 405nm. Soluble L-selectin was detected on the DREG-55-coated trapping plate, but not on the EL-246- or CL37-coated plates (Exhibit C - Figure 1). Signals for the EL-246-trapping MAb were actually less than control values. These results indicated that EL-246 cannot trap soluble L-selectin.

16. To exclude the possibility that the lack of apparent reactivity was due to the ELISA methodology, the ability of EL-246 MAb to directly immunoprecipitate soluble L-selectin was examined. COS cells were transiently mock transfected (Exhibit D - Figure 3, lanes 1, 4, and 7) or transfected with the L-selectin cDNA encoding the soluble form of L-selectin (Exhibit D - Figure 3 - lanes 2, 3, 5, 6, 8, and 9). The cells were metabolically labelled with [^{35}S]-methionine. Cell-free supernatants were collected after a 30 minute incubation at 37°C. The supernatants were analyzed for the presence of shed, soluble L-selectin by immunoprecipitation using DREG-56 or EL-246 monoclonal antibodies.

Specifically bound material was eluted with SDS sample buffer and resolved on an SDS-polyacrylamide gel. The gels were subjected to fluorography, dried and exposed to film. The autoradiograms were intentionally over-exposed to bring out weak bands. The results show that the DREG-56 antibody (Exhibit D - lane 3), but not the EL-246 antibody (Exhibit E - lane 6), immunoprecipitated the 68 kD soluble form of L-selectin from the cell-free supernatant of L-selectin transfectants.

Since we had prior evidence that EL-246 did not recognize the soluble form of L-selectin, some of the cells were pretreated with EL-246 MAb (Exhibit D - lanes 2, 5, 8) to determine whether allowing the EL-246 MAb to react with the cell surface form of L-selectin would allow the MAb to remain attached to L-selectin once L-selectin was shed. No EL-246-reactive material was immunoprecipitated with protein G-sepharose (Exhibit D - lane 8 compared with mock transfectants, lane 7, and untreated L-selectin transfectants, lane 9). Pretreatment of cells with EL-246 did not inhibit shedding of L-selectin or its immunoprecipitation by DREG-56 MAb (Exhibit D - lane 2).

17. The results provided herein conclusively show that the antibodies of the present invention, as exemplified by EL-246, do not bind to the shed or soluble form of L-selectin.

18. The immunogen disclosed in Kishimoto PNAS used to elicite the DREG monoclonal antibodies is the shed form of L-selectin. This immunogen would not result in the generation of antibodies disclosed and claimed in the present invention.

19. The monoclonal antibody of the present invention are novel and nonobvious and could not have been predicted based on the disclosure of Kishimoto et al <u>PNAS</u>.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made by information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 9/1/95

By: [signature]
Takashi Kei Kishimoto, Ph.D.

Curriculum Vitae

Takashi Kei Kishimoto, Ph.D.

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Education

1988	Ph.D. in Immunology Harvard University, Cambridge, Massachusetts
1983	B.A. in Biology New College of the University of South Florida, Sarasota, Florida

Professional Experience

1992 - Present: Principal Scientist, Department of Immunology
Boehringer Ingelheim Pharmaceuticals, Inc.
Ridgefield, Connecticut

Coordinate multidisciplinary research program in selectin adhesion molecules.

1990 - 1992 Senior Scientist, Department of Immunology
Boehringer Ingelheim Pharmaceuticals, Inc.
Ridgefield, Connecticut

Initiated research program at BIPI on selectin adhesion molecules

1988 - 1990 Postdoctoral Research (Dr. Eugene Butcher)
Stanford University, Stanford, California

1985 - 1988 Graduate Thesis Research (Dr. Timothy Springer)
Harvard University, Boston, Massachusetts

1984 Graduate Research Rotations
Harvard University, Boston, Massachusetts

1982 - 1983 Undergraduate Thesis Research
University of Maryland Cancer Research Center
Baltimore, Maryland

Teaching Experience

1991	Lecturer, Cellular adhesion molecules New York Medical College, Valhalla, N.Y.
1982-1983	Teaching Assistant, Genetics New College, Sarasota, Florida

Honors

1988 - 1990	Damon Runyon - Walter Winchell Cancer Foundation Fellowship
1983 - 1988	Albert J. Ryan Foundation Fellowship
1983 - 1986	National Science Foundation Predoctoral Fellowship
1986	Richard K. Smith Award for Outstanding Scientific Achievement by a Predoctoral or Graduate Fellow of the Dana-Farber Cancer Institute
1978 - 1979	New College Foundation Scholarship

Professional meetings organized

1991	Co-chairman, Lectin Cell Adhesion Molecules, New York Academy-Biochemical Pharmacology Discussion Group, New York
1991	Co-organizer, Second International Symposium on Structure and Function of Molecules Involved in Leukocyte Adhesion, Titisee, Germany
1995	Co-organizer, First International Ringberg Symposium on Molecular Mechanisms of Inflammation , Tegernsee, Germany

Professional Society Memberships

Leukocyte Biology Society
American Society for Investigative Pathology

Publications

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2. Sastre, L., T.K. Kishimoto, C. Gee, T.M. Roberts, and T.A. Springer. 1986. The mouse leukocyte adhesion proteins Mac-1 and LFA-1: Studies on mRNA translation and protein glycosylation with emphasis on Mac-1. J. Immunol. 137: 1060.

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9. Corbi, A.L., T.K. Kishimoto, L.J. Miller, and T.A. Springer. 1988. The human leukocyte adhesion glycoprotein Mac-1 (complement receptor type 3, CD11b) alpha subunit: Cloning, primary structure, and relation to the integrins, von Willebrand factor, and factor B. J. Biol. Chem. 263: 12403.

10. Kishimoto, T.K., K. O'Connor, and T.A. Springer. 1989. Leukocyte adhesion deficiency: Aberrant splicing of a conserved integrin sequence causes a moderate deficiency phenotype. J. Biol. Chem. 264: 3588.

11. Kishimoto, T.K., M.A. Jutila, E.L. Berg, and E.C. Butcher. 1989. The neutrophil Mac-1 and MEL-14 adhesion proteins are inversely regulated by chemotactic factors. Science 245: 1238.

12. Kishimoto, T.K. and T.A. Springer. 1989. Human leukocyte adhesion deficiency: Molecular basis for a defective immune response to infections of the skin. Curr. Probl. Dermatol. 18: 106.

13. Kishimoto, T.K., R.S. Larson, A.L. Corbi, M.L. Dustin, D.E. Staunton, and T.A. Springer. 1989. The leukocyte integrins. Adv. Immunol. 46: 149.

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15. Kishimoto, T.K., M.A. Jutila, and E.C. Butcher. 1990. Identification of a human peripheral lymph node homing receptor: A rapidly down-regulated adhesion molecule. Proc. Natl. Acad. Sci. U.S.A. 87: 2244.

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18. Hallmann, R., M.A. Jutila, C.W. Smith, D.C. Anderson, T.K. Kishimoto, and E.C. Butcher. 1991. The peripheral lymph node homing receptor, LECAM-1, is involved in CD18-independent adhesion of human neutrophils to endothelium. Biophys. Biochem. Res. Commun. 174: 236.

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